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TECHNICAL MANUSCRIPT 126

A SELECTIVE MEDIUM
FOR THE ISOLATION
OF PASTEURELLA PESTIS

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TECHNICAL MANUSCRIPT 126

A SELECTIVE MEDIUM FOR THE ISOLATION OF PASTEURELLA PESTIS

Ralph F. Knisely

Lois M. Swaney

Harold Friedlander

Physical Defense Division
DIRECTOR OF MEDICAL RESEARCH

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ABSTRACT

Several selective media are described that were successfully used to isolate virulent and avirulent strains of Pasteurella pestis from material heavily contaminated with other organisms. These media are comparatively easy to prepare, consist of readily available ingredients, and usually require no adjustment of the pH. One of the selective media described permits excellent recovery and the growth of large, easily distinguishable colonies of P. pestis in 48 hours at 26°C, and also allows the detection of fewer numbers of P. pestis organisms in soil than a previously recommended selective medium. The inhibition of other organisms frequently present in clinical specimens is described.

CONTENTS

Abstract	3
I. INTRODUCTION	7
II. MATERIALS AND METHODS	7
A. Bacterial Strains	7
B. Preparation of Cells	7
C. Antibiotics, Chemicals, and Culture Media	7
D. Selective Media	8
E. Preparation of Fildes' Digest	9
F. Survey for Selective Ingredients	9
G. Soil Studies with <u>P. pestis</u>	9
H. Identification of <u>P. pestis</u> Colonies	9
III. RESULTS	10
A. Survey for Selective Ingredients	10
B. Recovery of <u>P. pestis</u> on Plain Azide, Bile Salts and Morris Media	10
C. Recovery of <u>P. pestis</u> on Enriched Azide and Morris Media	10
D. Recovery of <u>P. pestis</u> from Soil Samples	10
E. Inhibition of Other Contaminants	15
F. Statistical Analysis	15
IV. DISCUSSION	16
Literature Cited	19

FIGURES

1. Recovery of <u>P. pestis</u> Strain A1122 (CD337) and Alexander Strain (CD404B) on Enriched Azide, Plain Azide, and Morris Medium after 48 Hours of Incubation	12
2. Relative Inhibition of Soil Organisms on Enriched Azide, Plain Azide, and Morris Medium	13

TABLES

I. Composition of Selective Media	8
II. Recovery of <u>P. pestis</u> after 72 Hours of Incubation (26°C) on Three Selective Media	11

III.	Recovery of <u>P. pestis</u> after 48 Hours of Incubation (26°C) on Enriched Azide and Morris Media	11
IV.	Average Recovery of Avirulent <u>P. pestis</u> (A1122) and Soil Organisms from Soil A on Plain Azide and Morris Media	14
V.	Recovery of Virulent <u>P. pestis</u> Alexander Strain and Soil Organisms from Soil B on Enriched Azide and Morris Media	14

I. INTRODUCTION

A selective medium for the isolation of Pasteurella pestis from material heavily contaminated with other organisms has been described by Morris.¹ Initial studies in this laboratory with the Morris medium showed marked variability with respect to recovery of virulent and avirulent strains as well as with individual strains in each group.

Our study is primarily concerned with the development of an azide medium that permits excellent recovery and growth of P. pestis in 48 hours from material heavily contaminated with other organisms. Test media evaluated in this study were (a) plain azide, (b) enriched azide, (c) Morris medium, and (d) blood agar base (BAB) as a control medium. The efficiency of a selective medium was evaluated on the basis of maximal recovery and growth of P. pestis organisms from known concentrations of test inocula. In addition, the effectiveness of the medium was also determined by the extent of inhibition of soil and other common contaminating organisms. The ease of preparation and the availability of ingredients were also important considerations.

II. MATERIALS AND METHODS

A. BACTERIAL STRAINS

Virulent and avirulent strains of P. pestis were employed in quantitative recovery experiments. The virulent strains were assayed for the proportion of avirulent cell types by the oxalate plate method of Higuchi and Smith.²

B. PREPARATION OF CELLS

Test inocula of each strain were prepared by suspending the growth (24 hours at 26°C) from two blood agar base slants in 10 milliliters of 0.06M phosphate buffer of pH 7.3. Dilutions containing approximately 10^8 organisms per milliliter were prepared for plating on selective media. Triplicate plates were inoculated with 0.1 milliliter of suspension and incubated at 26°C.

C. ANTIBIOTICS, CHEMICALS, AND CULTURE MEDIA

All culture media, potassium tellurite, and bile salts No. 3 were obtained from Difco. The following antibiotics and chemicals were obtained from the sources indicated. erythromycin (erythromycin lactobionate),

Abbot; novobiocin (Albamycin), Upjohn; ethyl violet, National Aniline Division of Allied Chemical and Dye Corporation; actidione, Upjohn; mycostatin, E.R. Squibb; hemin, Eastman Organic Chemicals; sodium azide, Eastman Kodak Company.

Antibiotic solutions were freshly prepared. Sterile solutions of other compounds were prepared and stored at 4°C. Hemin was dissolved in 0.05N NaOH and autoclaved. Calcium ion was added as $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$. Mycostatin was suspended in 70 per cent alcohol or dissolved in propylene glycol.

D. SELECTIVE MEDIA

The composition of selective media is presented in Table I. Ingredients were added after autoclaving the base agar and cooling to 45°C. Tryptose agar was employed in the Morris medium; BAB was used with the other media. The Morris medium was adjusted to pH 7.6 with 2N NaOH. The pH of the remaining media after addition of supplements was approximately pH 6.9 (unadjusted). Test media were generally used 24 hours after preparation, but satisfactory results could be obtained after storage for one week at 4°C.

TABLE I. COMPOSITION OF SELECTIVE MEDIA

	Base Medium	Erythro-mycin	Concentration, $\mu\text{g}/\text{ml}$				
			Ethyl Violet	Actidione	Mycostatin	Sodium Azide	Hemin
Plain Azide	BAB	40	1	100	100	7	--
Bile Salts ^{a/}	BAB	40	1	100	100	--	--
Enriched Azide ^{b/}	BAB	40	1	100	100	5	40
Morris Medium ^{c/}	Tryptose Agar	5	--	100	--	--	--

- 1500 micrograms of bile salts added per milliliter.
- Additions to enriched azide medium consisted of 0.1 per cent glucose and 0.1 M Ca^{++} as $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$.
- Supplements to the Morris medium consisted of 10 micrograms of novobiocin per milliliter; 5 micrograms of potassium tellurite per milliliter and 5 per cent (v/v) peptic digest of sheep blood (medium adjusted to pH 7.6; remaining media are unadjusted at pH 6.9).

E. PREPARATION OF FILDES' DIGEST

Fresh sheep blood was obtained aseptically and defibrinated with glass beads. The method of Fildes³ was followed using Difco (N.F.) pepsin, and the digestion was allowed to proceed with occasional shaking for approximately five hours in a water bath at 55°C.

F. SURVEY FOR SELECTIVE INGREDIENTS

Nineteen virulent and 15 avirulent strains of P. pestis were tested for sensitivity to various antibiotics and dyes. Sensitivity discs (Difco) were employed for antibiotic studies. Antibiotics that failed to inhibit P. pestis were tested for their effect on soil organisms. A variety of concentrations and combinations were evaluated at various pH levels.

G. SOIL STUDIES WITH P. PESTIS

Soil was considered as representing a specimen heavily contaminated with other bacteria. Comparative studies were conducted utilizing plain and enriched azide media, bile salts medium, and Morris medium. A local soil sample (Soil A) and a garden sample (Soil B) were employed in these experiments. Each soil was passed through screens of decreasing pore size to remove large clumps and other debris. The moisture content was not adjusted for this experiment. One-gram samples of soil were inoculated with the test strain and suspended in a total volume of 10 milliliters of phosphate buffer (pH 7.3). Suspensions or dilutions were vigorously shaken and 0.1 milliliter was plated on triplicate plates. For plating on BAE control plates, 0.1 milliliter of a 10^{-8} dilution of the suspension was employed.

H. IDENTIFICATION OF P. PESTIS COLONIES

Colonies on plates were carefully examined with a stereoscopic microscope (12X), using oblique illumination. Representative colonies possessing typical morphology of P. pestis were subcultured on tryptose agar for more detailed study.

III. RESULTS

A. SURVEY FOR SELECTIVE INGREDIENTS

Extensive testing showed that a selective medium for P. pestis could be prepared by utilizing blood agar base in combination with sodium azide, erythromycin, actidione, mycostatin and ethyl violet. The addition of hemin improved the recovery of P. pestis on this medium. Optimal results were obtained by lowering the sodium azide concentration and adding glucose and calcium ion.

B. RECOVERY OF P. PESTIS ON PLAIN AZIDE, BILE SALTS AND MORRIS MEDIA

Recovery efficiencies after 72 hours of incubation on plain azide, bile salts, Morris and BAB media are compared in Table II. Blood agar base served as the control medium. Maximum recovery of all strains was generally obtained on the Morris and BAB media in 48 hours; a period of 72 hours was required with the other media. Atypical colonies of P. pestis were produced on the bile salts medium. A higher recovery was obtained with bile salts and plain azide media than with the Morris medium.

C. RECOVERY OF P. PESTIS ON ENRICHED AZIDE AND MORRIS MEDIA

Recovery with enriched azide media in 48 hours was markedly superior to that with the Morris medium (Table III). In addition, the recovery rates were improved over those obtained with plain azide media (Table II). Maximum recovery on enriched azide was obtained in 48 hours. The colonies were large and possessed characteristic morphology.

Figure 1 shows the relative size and numbers of a virulent (Alexander) and avirulent (A1122) strain of P. pestis on enriched azide and Morris media.

D. RECOVERY OF P. PESTIS FROM SOIL SAMPLES

The recovery of avirulent P. pestis (A1122) was higher on plain azide than on the Morris medium utilizing 700 and 1400 cells per gram of soil (Table IV). Similar results were obtained with enriched azide and the virulent Alexander strain (Table V). Figure 2 shows growth of soil organisms from soil suspensions A and B on plain and enriched azide and the Morris medium. In order to recover P. pestis from Soil A and B by plating on BAB media, it was necessary to use an inoculum of 2.5×10^5 and 1×10^5 P. pestis cells per gram of soil, respectively.

TABLE II. RECOVERY OF P. PESTIS AFTER 72 HOURS OF INCUBATION (26°C)
ON THREE SELECTIVE MEDIA

Test Strains ^{b/}	Per Cent Recovery ^{a/}			Control (BAB)
	Plain Azide Medium ^{c/}	Bile Salts Medium ^{c/}	Morris Medium ^{c/}	
14 avirulent	80	96	29	100
14 virulent	85	85	61	100

- a. Plate counts obtained by averaging three plates for each strain.
 b. Avirulent: A-12, Tjiwidej, Soemedang, EV-76, A1122, TRU, A-4, Java, 556a, M23B, A-16, 53-H-1, M33, and AD5-S.
 Virulent: Alexander, Charleston, Kuma, Washington, Yokohama, M41-1, Powell, MPG, Shasta, M23, Saka, 556, H5, and H15.
 c. Range of recovery was as follows: Plain azide medium - not less than 58 per cent recovery except for one avirulent strain (4 per cent) and one virulent strain (25 per cent); Bile salt medium - not less than 50 per cent recovery for all strains; Morris medium - less than 50 per cent recovery for 11 avirulent strains and 3 virulent strains.

TABLE III. RECOVERY OF P. PESTIS AFTER 48 HOURS OF INCUBATION
(26°C) ON ENRICHED AZIDE AND MORRIS MEDIA

Test Strains ^{b/}	Per Cent Recovery ^{a/}		Control (BAB)
	Enriched Azide Medium ^{c/}	Morris Medium ^{c/}	
14 avirulent	95	27	100
12 virulent	105	39	100

- a. Plate counts obtained by averaging three plates for each strain.
 b. Avirulent: A-12, Tjiwidej, Soemedang, EV-76, A1122, TRU, A-4, Java, 556a, M23B, A-16, M-33, A1224, and AD5-S.
 Virulent: Alexander, Charleston, Kuma, Washington, Yokohama, Powell, M41-1, MPG, Shasta, M23, Saka, and 556.
 c. Recovery on enriched azide medium was not less than 74 per cent for any strain. Recovery of the avirulent strains on Morris medium ranged from 0 to 57 per cent and recovery of the virulent strains from 0 to 80 per cent.

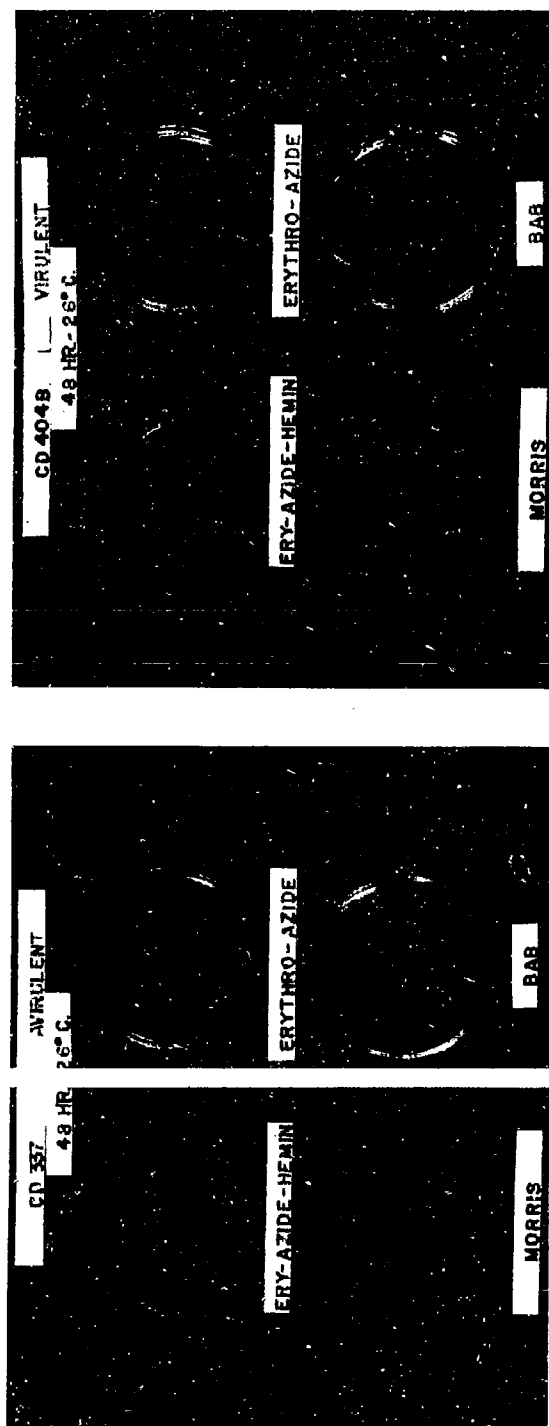


Figure 1. Recovery of *P. pestis* Strain A1122 (CD337) and Alexander Strain (CD404B) on Enriched Azide (Top, Left) and Plain Azide (Top, Right) and Morris Medium after 48 Hours of Incubation. The BAB plate serves as a control.

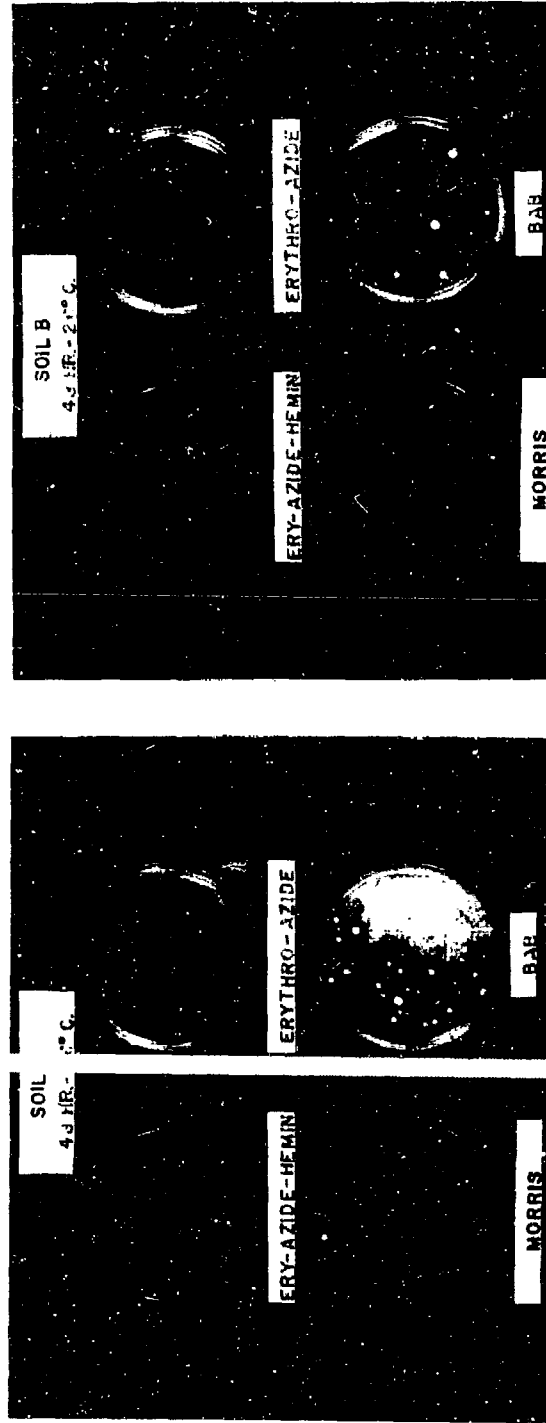


Figure 2. Relative inhibition of Soil Organisms on Enriched Azide (Top, Left), Plain Azide (Top, Right), and Morris Medium. The selective media were inoculated with 0.1 milliliter of a 10^{-1} dilution of soil suspension. The BAB was inoculated with 0.1 milliliter of a 10^{-3} dilution of soil suspension. Note the spreading-type colony or the BAB plate of soil A.

TABLE IV. AVERAGE RECOVERY OF AVIRULENT P. PESTIS (A1122)
AND SOIL ORGANISMS FROM SOIL A ON PLAIN AZIDE
AND MORRIS MEDIA^{a/}

Initial Inoculum of <u>P. pestis</u> Cells Per Gram of Soil	Dilution of Soil Suspension Plated	Average Recovery of <u>P. pestis</u>		Average Recovery of Soil Organisms	
		Plain Azide	Morris	Plain Azide	Morris
700	Undiluted ^{b/}	7.9	2.3	202	321
	Undiluted	2.4	0.8	103	117
	1:2	2.9	0.9	49	50
1400	Undiluted	6.8	2.9	185	192
	1:2	3.6	1.5	90	87
	1:5	1.9	0.7	45	35

- a. Counts obtained from three samples (triplicate plates averaged).
b. 0.2 milliliter of undiluted suspension plated; 0.1 milliliter quantities plated from all other suspensions and dilutions.

TABLE V. RECOVERY OF VIRULENT P. PESTIS ALEXANDER STRAIN
AND SOIL ORGANISMS FROM SOIL B ON ENRICHED AZIDE
AND MORRIS MEDIA^{a/}

Initial Inoculum of <u>P. pestis</u> Cells Per Gram of Soil	Dilution of Soil Suspension Plated	Average Recovery of <u>P. pestis</u>		Average Recovery of Soil Organisms	
		Enriched Azide	Morris	Enriched Azide	Morris
595	Undiluted	4.7	0.9	47	23
	1:2	2.8	0.4	26	16
1190	Undiluted	8.0	1.7	22	15
	1:2	3.8	0.9	10	8

- a. Counts obtained from three samples (triplicate plates averaged).

E. INHIBITION OF OTHER CONTAMINANTS

The following organisms were inhibited on plain azide media and the Morris medium: Aerobacter aerogenes, Alcaligenes faecalis ATCC-8750, Escherichia coli ATCC-11303, Escherichia coli B, Pasteurella mastidis ATCC-10899, Pasteurella multocida ATCC-9656, Shigella sonnei 16, Streptococcus faecalis, and Vibrio comma VM-7708. The enriched azide media inhibited all these strains except Aerobacter aerogenes, E. coli B, and Shigella sonnei. Recovery of E. coli on this medium was 56 per cent and of Shigella sonnei 49 per cent of the recovery on the BAB control.

Paracolobactrum coliforme, Proteus vulgaris, and Salmonella typhosa A-60 were inhibited on the Morris medium but not on the plain or enriched azide media. Recovery of six strains of Pasteurella pseudotuberculosis (ATCC 6902, 6903, 6904, 6905, 913, and 11960) averaged 74 per cent on plain azide, 94 per cent on enriched azide, and 45 per cent on the Morris medium. Pseudomonas aeruginosa recovery was 100 per cent on plain azide, 90 per cent on enriched azide, and 22 per cent on the Morris medium. All percentages are in comparison with recovery on BAB plates. Strains not specifically identified were stock strains that had been used for student instruction.

F. STATISTICAL ANALYSIS

Analyses of variance were performed and comparisons made at the five per cent probability level. The statistical analysis of the data showed that the recovery of virulent and avirulent strains of P. pestis was significantly higher on plain and enriched azide and bile salts media than on the Morris medium (Tables II and III). The recovery of P. pestis from Soil A (Table IV) on plain azide was significantly higher than on the Morris medium for both levels of inoculum. The analysis indicated that there was no significant difference between these two selective media in the inhibition of the soil organisms from Soil A.

The analysis of the data in Table V showed that there was a significantly higher recovery of P. pestis on the enriched azide medium compared with that on the Morris medium. There was no significant difference in inhibition of soil organisms by either of the selective media.

IV. DISCUSSION

The Morris medium was difficult to prepare, required adjustment of the pH, and did not allow for efficient recovery of P. pestis. Recovery on this medium prepared in our laboratory varied from 27 per cent for the avirulent strains to 60 per cent for the virulent strains when compared with blood agar base controls. The recovery of individual strains of P. pestis frequently varied each time the medium was prepared.

Markenson and Ben-Efraim⁴ describe an oxgall medium for identification of P. pestis. This medium requires two per cent defibrinated rabbit blood. Preliminary results with this medium indicate that it is less inhibitory for contaminating organisms than azide media. Recovery of 12 virulent strains of P. pestis averaged 56 per cent compared with recovery on BAB. This medium also has the disadvantage of being opaque.

Previous attempts by other investigators to isolate P. pestis on selective media from various specimens were not entirely satisfactory.⁵⁻⁷ A medium for the cultivation of Pasteurella and Brucella species utilizing brilliant green as the inhibitory agent was described by Levin, Trupin, and Cabelli.⁸ This medium requires the use of a peptic digest of bovine hemoglobin.

The bile salts medium permitted good recovery of P. pestis, but colony morphology was atypical. Similar variations in colony morphology on a bile salts medium were described by LaRose.⁹ Recognition of P. pestis colonies among soil colonies on this medium was more difficult than with other selective media. The bile salts medium is therefore recommended only in special situations where the contaminants are particularly sensitive to bile salts. The use of bile solution as an enrichment fluid for the isolation of P. pestis was reported by Kirschner.¹⁰

The plain azide medium requires 72 hours for recovery of P. pestis, but has the advantage of being more inhibitory than the enriched azide medium for certain types of contaminating organisms. The enriched azide medium has an advantage of allowing excellent recovery of P. pestis in 48 hours and growth of large easily distinguishable colonies.

Hemin served as a satisfactory enrichment in the azide medium. It eliminated the necessity of obtaining sheep blood and the time required to prepare the digest. Herbert¹¹ reported that hematin was the factor in pepsinized blood responsible for increased growth of P. pestis in agar media.

The correlation of virulence of P. pestis with calcium ion was reported by Higuchi, Kupferberg, and Smith¹² and Higuchi and Smith.² Glucose and calcium ion were added in an attempt to improve the azide medium. Although

the addition of calcium and glucose does not significantly increase the recovery of P. pestis, it does aid in obtaining large, uniform colonies possessing a characteristic morphology. The advantages of the proposed azide media are easy preparation, utilizing readily available ingredients, and the possibility of direct isolation of P. pestis from heavily contaminated material. The growth of many organisms frequently encountered in clinical specimens is inhibited on these media.

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